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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

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INTERNATIONAL APPLICATION NO.  
PCT/US98/04599INTERNATIONAL FILING DATE  
09 MARCH 1998 (09.03.98)PRIORITY DATE CLAIMED  
10 MARCH 1997 (10.03.97)

TITLE OF INVENTION

METHOD FOR THE IDENTIFICATION OF GENETIC SUBTYPES

APPLICANT(S) FOR DO/EO/US  
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☒ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

## Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.  
A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☒ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☐ Other items or information:

17. A general power of attorney

18. Mailing Express Label No.: EJ368938536US

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510 Rec'd PCT/PTO n 1 SEP 1999

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20. The following fees are submitted.:

CALCULATIONS PTO USE ONLY

**BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5) ) :**

- ☒ Search Report has been prepared by the EPO or JPO ..... \$840.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... \$670.00
- ☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... \$760.00
- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$970.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... \$96.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

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CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	27 - 20 =	7	x \$18.00
Independent claims	2 - 3 =	0	x \$78.00

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Multiple Dependent Claims (check if applicable). ☐

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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☐

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- ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **04-1928** A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

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TITLEMETHOD FOR THE IDENTIFICATION OF GENETIC SUBTYPESFIELD OF INVENTION

The present invention relates to the field of microbiology and methods for  
5 identification of genetic subtypes within a given genetic population. More specifically the process involves the method of Restriction Fragment Length Polymorphism (RFLP) using frequent cutting restriction endonucleases (RE) to generate a genetic fingerprint distinctive of a specific genetic subtype.

BACKGROUND

10 Infectious diseases are a prominent health hazard in industrial as well as developing countries. For example, two to three million cases of *Salmonella* food poisoning are estimated to occur each year in the United States. Acute symptoms of this disorder include nausea, vomiting, diarrhea, cold chills, fever, and exhaustion. About 2,000-3,000 (0.1%)  
15 people die each year in the United States from *Salmonella* poisoning, these victims usually including infants, the sick, and elderly.

Poultry, red meat, seafood, eggs and any foods which contain these products particularly have the potential to carry bacterial pathogens. The challenge to an epidemiologist dealing with infectious diseases, is to be able to discern, not only which  
20 pathogen is responsible for the disease, but also which serotype or subtype of the pathogen is present. The ability to subtype the organisms causing a particular disease offers the best chance of determining the source of the outbreak and controlling the spread of the disease.

As methods of molecular subtyping expand in epidemiological investigations of infectious diseases there is a corresponding need for universal subtyping methods. Several methods of genetic subtyping are practiced, including macro-restriction fragment length  
25 polymorphism (Ma-RFLP) and PCR based methods.

Ma-RFLP methods involve the use of restriction endonucleases that cut DNA infrequently (rare cutters) which results in the generation of a discreet number of relatively large DNA fragments. These fragments are resolved by gel electrophoresis where the  
30 banding pattern on the gel is used to differentiate discrete strains and subtypes within a population of microorganisms. The DNA fragment size produced in Ma-RFLP is generally on the order of 10-1000 kilobase pairs (kb).

Ma-RFLP has been used effectively in epidemiological studies for the subtyping of organisms. For example, the method has been used to differentiate strains and subtypes of *Listeria* [Nesbakken et al., *International Journal of Food Microbiology* 31 (1-3), 1996,  
35 161-171; Saito et al., *Japanese Journal of Medical Science and Biology* 50 (2), 1997, 63-71.; Howard et al., *Appl. Environ. Microbiol.* (1992), 58(2), 709-12;] *Salmonella*, [Kapperud et al., *J Clin Microbiol* 27 (9), 1989, 2019-2024.; Koh-Luar et al., *World Journal of*

*Microbiology & Biotechnology* 12 (4), 1996, 405-407] *Campylobacter*, [Owen et al., *J Clin Microbiol* 27 (10), 1989, 2338-2343], and *Pseudomonas* [Goldstein, R., WO 9701647].

sss

Although Ma-RFLP is the current method of choice for subtype determination the method is labor-intensive, cumbersome, takes 2-5 days to perform, and requires expensive electrophoretic equipment such as pulsed field gel electrophoresis (PFGE) apparatus for resolving the DNA fragments. Additionally the resolution of gel bands is often poor, making interpretation of the data suspect.

Another commonly used method of genetic subtyping employs polymerase chain reaction (PCR) amplification of genomic or chromosomal DNA either alone or in combination with restriction analysis or probe hybridization. Typically PCR primers, specific for a particular organism, are used to amplify a specific portion of the genome, which is then restricted using appropriate restriction enzymes. For example PCR based genetic subtyping has been used to analysis of populations of *Mycobacterium*, [Haas et al., *Journal of Clinical Microbiology* 35 (3), 1997, 663-666, (epidemiological tracking of *M. africanum* and *M. tuberculosis*)], and *Salmonella*, [Burr et al., *J. Microbiol. Methods* (1997), 29(1), 63-68, (distinguishing *Salmonella* and *E. coli* serotypes); Lin et al., *J. Clin. Microbiol.* (1996), 34(4), 870-6, (differentiation of *S. enteritidis* isolates)].

PCR based methods, while effective in differentiating some genetic subtypes, generally require advanced knowledge of the genome for the generation of specific primers, making the method less applicable to situations where little is known about the subject organism.

A method termed small fragment restriction endonuclease analysis (SF-REA) has been developed and has been used for epidemiological studies [Haertl et al., *Zentralbl. Bakteriol.* (1994), 280(3), 312-18]. This technique relies on the use of infrequent cutting restriction endonucleases and analyzes the fragments generated in the 0.5 to 4 kb range. Restriction enzymes with 6-base recognition sequences are used to generate the RFLP pattern. The RFLP pattern range starts at 20-40 kb, and is virtually impossible to decipher. Polyacrylamide gel separation is necessary to achieve effective separation in the lower size range which has very limited use for interstrain differentiation.

Yet another method for genetic subtyping has been developed involving the application of restriction analysis to chromosomal DNA. The method is termed chromosomal restriction endonuclease analysis (CREA) and has been applied to various prokaryotic genomes with only moderate success. For example, Tsai et al., (*Gyobyo Kenkyu* (1990), 25(4), 201-6) disclose the restriction of genomic DNA isolated from the fish pathogens *Vibrio alginolyticus*, *V. anguillarum*, *V. damsela*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, and *V. vulnificus* with a variety of restriction enzymes and resolution

of fragments ranging from 2 kb to 23 kb by gel electrophoresis to obtain a characteristic fingerprint for each pathogen. Only 6 base specific restriction endonucleases were effective in generating specific patterns.

For the most part RFLP subtyping has employed rare cutters (infrequent cutters) in order to generate a manageable number of fragments. There are a few reports where more frequent cutters are used, with minimal success. For example, Preston et al. (*J. Clin. Microbiol.* (1994), 32(6), 1427-30) describe a method for the RFLP analysis of 49 clinical isolates of *Shigella sonnei* with the four base cutting restriction endonucleases *HaeIII* and *RsaI*. Restriction fragments were resolved by gel electrophoresis and visualized by silver staining. Analysis revealed 15 distinct RFLP patterns by *HaeIII* digestion, and 12 distinct patterns by *RsaI* digestion. The authors note that it was not possible to detect the bands on the gel using a DNA binding dye and silver stain was substituted as a detection means. Additionally, polyacrylamide was taught as the only effective gel medium for this type of separation.

Similarly, Wesley, et al., (*Microb. Toxins Foods Feeds: Cell. Mol. Modes Action*, [Proc. Symp.] (1990), Meeting Date 1988, 225-38. Editor(s): Pohland, Albert E.; Dowell, Vulus R., Jr.; Richard, John L. Publisher: Plenum, New York, NY) teach the restriction of genomic DNA isolated from *L. monocytogenes* with the four base cutter *HhaI* and resolution of the resulting fragments by gel electrophoresis. Although a frequent base cutter was used, Southern blotting and hybridization with a synthetic oligomer probe specific for the beta-hemolysin gene was necessary for final resolution.

Like Wesley et al., Matsuura, et al., (*J. Helminthol.* (1992), 66(4), 261-6) teach the use of seven kinds of restriction endonucleases including 4-base cutters for the digestion of *Diphyllobothrium* ribosomal DNA and resolution of the fragments on a gel for the identification of species-specific patterns. Final differentiation of subtypes was made using a characteristic 25S rDNA probe. This method of subtyping by Southern blot hybridization with ribosomal RNA-based probes, commonly referred to as ribotyping, is well known in the scientific literature.

The above mentioned methods are useful for genetic subtyping but are limited by lack of specificity and efficiency of application. There is a need therefore for a method that provides the rapid, specific and accurate subtyping of genetic populations that does not rely on the cumbersome techniques of Ma-RFLP pulsed-field gel analysis.

The Applicant has provided the solution to the stated problem by developing a highly specific restriction enzyme based process that analyzes the DNA banding patterns of small DNA fragments produced by the use of appropriate restriction enzymes (mostly frequent base cutters) which yield banding patterns in the size range below 18 kb.

### SUMMARY OF THE INVENTION

The present invention provides a method for the differentiation of genetic sub-types within a population of genetically related organisms comprising the steps of:

- (i) isolating genomic DNA from an organism to be analyzed;
- 5 (ii) restricting an excess of genomic DNA from said organism with at least one restriction endonuclease to yield restricted DNA having fragments less than 18 kb;
- (iii) resolving said restricted DNA by chromatographic means to generate a distinct genetic fingerprint;
- (iv) comparing said distinct genetic fingerprint of step (iii) with the genetic
- 10 fingerprint of a specific genetic sub-type wherein differences in said genetic fingerprints afford differentiation of genetic sub-types within a population of genetically related organisms.

The invention further provides a method for the interstrain differentiation of bacterial isolates, comprising the steps of:

- 15 (i) isolating genomic DNA from a) a panel of epidemiologically linked bacterial isolates and b) a panel of epidemiologically un-linked bacterial isolates wherein said linked and un-linked isolates are members of the same genus;
- (ii) restricting an excess of genomic DNA from the linked and un-linked panel of isolates with a panel of restriction enzymes selected from the group consisting of *AccI*,  
 20 *AluI*, *AvaI*, *AvaII*, *BanII*, *BfaI*, *CfoI*, *DdeI*, *DpnI*, *HaeII*, *HaeIII*, *HhaI*, *HincII*, *HinfI*, *HpaII*,  
*MboI*, *MnII*, *MseI*, *NciI*, *NlaIV*, *RsaI*, *Sau3AI*, *ScrFI*, *TaqI*, and *ThaI*;
- (iii) resolving the restricted DNA of step (ii) by gel electrophoresis to generate a banding pattern for each enzyme;
- (iv) selecting at least one enzyme from the panel of enzymes of step (ii) that
- 25 a) generates DNA fragments less than 18 kb, b) generates the lowest index of diversity from the linked panel and c) generates the highest index of diversity from the unlinked panel;
- (v) restricting the isolated DNA from the linked and un-linked panels of bacterial isolates of step (i) with the at least one enzyme of step (iv);
- (vi) resolving said restricted DNA of step (v) by gel electrophoresis to generate
- 30 a distinct genetic fingerprint for said linked and unlinked isolates; and
- (vii) comparing said distinct genetic fingerprints of step (vi) wherein differences in said genetic fingerprints affords interstrain differentiation of said bacterial isolates.

### BRIEF DESCRIPTION OF THE FIGURES

Figure 1a is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction  
 35 endonuclease *AscI*, *DdeI*, *DpnII* or *MnII*.

Figure 1b is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *AscI*, *AvaI*, or *BfaI*.

Figure 1c is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *TaqI*, *Sau3AI*, *NciI* or *AluI*.

Figure 1d is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *MspI* or *HhaI*.

5 Figure 1e is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *ScrFI* or *RsaI*.

Figure 1f is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *BstUI*.

10 Figure 1g is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *AvaII*.

Figure 1h is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *HinfI*.

Figure 1i is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *NlaIV*.

15 Figure 1j is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *PvuII*, *HindIII* or *HincII*.

Figure 1k is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *EcoRI*, *BspI* or *BamHI*.

20 Figure 1l is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *HaeIII*.

Figure 1m is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *NlaIII*.

Figure 2a is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *RsaI*, *ScrFI* or *Sau3AI*.

25 Figure 2b is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *HhaI* or *MspI*.

Figure 2c is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *NlaIV*.

30 Figure 2d is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *HhaI*.

Figure 2e is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *HaeIII*.

Figure 2f is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *Sau3AI*.

35 Figure 2g is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *AluI*.

Figure 2h is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *NciI*.

Figure 2i is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *AluI*, *NciI*, *Sau3AI* or *HaeIII*.

5 Figure 3a is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *HaeIII*.

Figure 3b is an agarose Gel of *E. coli* O157:H7 DNAs digested with restriction endonuclease *Sau3AI*.

10 Figure 4 is an agarose Gel of *E. coli* O157:H7 DNAs (strains 112, 2096, 2160) digested with restriction endonuclease *PvuII*, *HindIII*, *EcoRI* or *Sau3AI*.

Figure 5 is an agarose Gel of *E. coli* O157:H7 (strain 112) DNA double-digested with restriction endonucleases *HincII* and *SmaI*, *PstI* and *HindIII* or *EcoRV* and *HindIII*.

Figure 6 is an agarose Gel of increasing masses of *E. coli* O157:H7 (strain 112) DNA digested with restriction endonuclease *HaeIII* or *Sau3AI*.

15 Figure 7a is an agarose gel (2%) of *NciI* or *Sau3AI* digested DNA from three strains of *E. coli* O157:H7.

Figure 7b is an agarose gel (1%) of *NciI* or *Sau3AI* digested DNA from three strains of *E. coli* O157:H7.

20 Figure 8a is an agarose gel (0.7%) of *Sau3AI* digested DNA from three strains of *E. coli* O157:H7.

Figure 8b is an agarose gel (0.8%) of *Sau3AI* digested DNA from three strains of *E. coli* O157:H7.

Figure 8c is an agarose gel (1.0%) of *Sau3AI* digested DNA from three strains of *E. coli* O157:H7.

25 Figure 9a is an agarose gel of *Shigella sonnei*, *Salmonella bovis-morbificans* and *E. coli* O157:H7 DNAs digested with *AvaI*, *BstUI*, *DpnII*, *HhaI*, *MspI* or *NlaIII* restriction endonucleases.

Figure 9b is an agarose gel of *Shigella sonnei*, *Salmonella bovis-morbificans* and *E. coli* O157:H7 DNAs digested with *NlaIV*, *RsaI* or *ScrFI* restriction endonucleases.

30 Figure 9c is an agarose gel of DNAs from three *Shigella sonnei* isolates digested with *Sau3AI*, *NciI*, or *HaeIII* restriction endonucleases.

Figure 9d is an agarose gel of DNAs from three *Salmonella bovis-morbificans* isolates digested with *Sau3AI*, *NciI*, or *HaeIII* restriction endonucleases.

35 Figure 10a is an agarose gel of *HaeIII*-digested DNAs from epidemiologically-linked and -unlinked *Shigella sonnei* isolates.

Figure 10b is an agarose gel of *Sau3AI*-digested DNAs from epidemiologically-linked and -unlinked *Shigella sonnei* isolates.



Figure 11a is an agarose gel of *HaeIII* digested DNAs from 11 *Salmonella bovis-morbificans* isolates of known epidemiologic history.

Figure 11b is an agarose gel of *AluI* digested DNAs from 11 *Salmonella bovis morbificans* isolates of known epidemiologic history.

5 Figure 11c is an agarose gel of *NciI* digested DNAs from 11 *Salmonella bovis-morbificans* isolates of known epidemiologic history.

Figure 11d is an agarose gel of *Sau3AI* digested DNAs from 11 *Salmonella bovis-morbificans* isolates of known epidemiologic history.

10 Figure 12a is an agarose gel of DNAs from three *Neisseria meningitidis* isolates digested with *Sau3AI*, *NciI*, or *HaeIII* restriction endonucleases.

Figure 12b is an agarose gel of DNAs from two *Neisseria meningitidis* isolates digested with *Sau3AI*, *HaeIII* or *MnII* restriction endonucleases.

Figure 12c is an agarose gel of DNAs from six *Pseudomonas aeruginosa* isolates digested with *NciI*, *Sau3AI*, or *HaeIII* restriction endonucleases.

15 Figure 12d is an agarose gel of *NciI* digested DNAs from 5 *Pseudomonas aeruginosa* isolates of known epidemiologic history.

Figure 13a is an agarose gel of *Sau3AI* or *HaeIII* digested DNAs from 7 *Klebsiella pneumonia* isolates of known epidemiologic history.

20 Figure 13b is an agarose gel of *HaeIII* digested DNAs from *Vibrio parahaemolyticus* isolates of known epidemiologic history.

Figure 13c is an agarose gel of *Sau3AI* digested DNAs from *Vibrio parahaemolyticus* isolates of known epidemiologic history.

Figure 14a is an agarose gel of *HaeIII* digested DNAs from *Enterococcus* species isolates of known epidemiologic history.

25 Figure 14b is an agarose gel of *HhaI* digested DNAs from *Enterococcus* isolates of known epidemiologic history.

Figure 14c is an agarose gel (0.6%) of *HaeIII* digested DNAs from *Enterococcus* isolates of known epidemiologic history.

30 Figure 15a is an agarose gel of *Staphylococcus aureus* DNA digested with the 13 different restriction endonucleases *AluI*, *AscI*, *AvaI*, *AvaII*, *BfaI*, *BstUI*, *DdeI*, *DpnII*, *MnII*, *MseI*, *MspI*, *RsaI* and *ScrFI*.

Figure 15b is an agarose gel of *HaeIII* digested DNAs from 10 *Staphylococcus aureus* isolates of known epidemiologic history.

35 Figure 15c is an agarose gel of *NlaIV* digested DNAs from 10 *Staphylococcus aureus* isolates of known epidemiologic history.

Figure 15d is an agarose gel of *HhaI* digested DNAs from 9 *Staphylococcus aureus* isolates of known epidemiologic history.

Figure 15e is an agarose gel of *AluI* digested DNAs from 7 *Staphylococcus aureus* isolates of known epidemiologic history.

Figure 15f is an agarose gel of *DdeI* digested DNAs from 7 *Staphylococcus aureus* isolates of known epidemiologic history.

5 Figure 15g is an agarose gel of *DdeI* and *MnlI* digested DNAs from 7 *Staphylococcus aureus* isolates of known epidemiologic history.

Figure 15h is an agarose gel of *HhaI* digested DNAs from 7 *Staphylococcus aureus* isolates of known epidemiologic history.

10 Figure 16a is an agarose gel of *Listeria monocytogenes* DNA digested with the 13 different restriction endonucleases *AluI*, *Ascl*, *AvaI*, *BfaI*, *BstUI*, *DdeI*, *DpnII*, *MnlI*, *MseI*, *MspI*, *NlaIII*, *RsaI* and *ScrFI*.

Figure 16b is an agarose gel of *DpnII* digested DNAs from 12 *Listeria monocytogenes* isolates of known epidemiologic history.

### DETAILED DESCRIPTION OF THE INVENTION

15 As used herein the following terms may be used for interpretation of the claims and specification.

The term "genetic subtype" means an individual microbial cell or group of microbial cells of a particular taxonomic genus, species, subspecies, serovar or strain which can be distinguished on the basis of differences in genomic DNA sequence or structure revealed by  
20 DNA sequencing, restriction fragment length polymorphism or other DNA-based methodology from other individual microbial cells or groups of microbial cells of the same taxonomic classification.

The terms "genetic subtyping" and "inter-strain differentiation" will be used interchangeably and will refer to any process for identifying a genetic subtype.

25 The term "epidemiological history" means information which relates microbial strains isolated from clusters of patients grouped on the basis of common exposure to a specific microbe. Discrete microbial isolates which have a known epidemiological history may or may not be linked to a common source or exposure.

30 The term "excess DNA" refers the amount of DNA use for restriction digest and will vary from about 5 ug to about 30 ug.

The term "chromatographic means" refers to any method of chromatographically separating nucleic acid fragments. Typical method include gel electrophoresis, high pressure liquid chromatography (HPLC), flow cytometry and membrane separation.

35 The term "banding pattern" means a pattern of DNA fragments distinctly separated on a gel or other chromatographic media.

The term "genetic fingerprint" means an array of DNA or RNA fragments or DNA or RNA nucleotide sequence from an organism which is sufficient to distinguish it from

another organism of the same taxonomic classification at the level of genus, species, subspecies, serovar or strain designation.

The term "microbial" means bacteria, fungi, and parasites.

5 The term "epidemiologically linked" means independently isolated strains (isolated from a group of patients who have been exposed to the same vehicle of infection) which belong to the same species and are of clonal origin. In non-medical setting the term would indicate a group of isolated microbes which are of clonal origin.

The term "epidemiologically unlinked" means a group of isolates which are not of clonal origin.

10 The term "restriction endonuclease" or "restriction enzyme" will be abbreviated as RE and will mean an enzyme which binds and cuts within a specific nucleotide sequence within double-stranded DNA.

15 The term "4 base cutter" means a restriction endonuclease which recognizes and requires a specific DNA sequence of four nucleotides for site-specific digestion of double-stranded DNA.

The term "6 base cutter" means a restriction endonuclease which recognizes and requires a specific DNA sequence of six nucleotides for site-specific digestion of double-stranded DNA.

20 The term "genetically related population" refers to any grouping of organisms possessing multiple or single phenotypic characteristics of sufficient similarity to allow said organisms to be classified as a single genus, species, or subspecies.

The term "index of diversity" means the number of discrete subtypes identified by the number of isolates analyzed by a specific subtyping method.

25 "Nucleic acid" refers to a molecule which can be single-stranded or double-stranded, comprising monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. In bacteria, lower eukaryotes, and in higher animals and plants, "deoxyribonucleic acid" (DNA) refers to the genetic material while "ribonucleic acid" (RNA) is involved in the translation of the information from DNA into proteins.

30 The present invention describes a method for the identification of genetic subtypes within a given related genetic population. The method utilizes the variation seen in a DNA banding pattern when genomic or chromosomal DNA is restricted with frequent base cutters. Only fragments between 0.1 and 18 kb are analyzed.

35 The present invention and its variations can be used for a myriad of applications. It may be used by plant or animal breeders to correctly identify their subjects, or it may be used by clinical and microbiological laboratories to identify bacteria, parasites or fungi present in any medium, including in eukaryotic cells. In this latter use, the method is preferred to the standard microbiological assays, since it does not require isolation and growth of the

microbes. In vitro growth and characterization is now either impossible for some microorganisms such as *Mycobacterium leprae* (agent of leprosy), impossible on standard media for some microorganisms such as the obligate intracellular bacteria (e.g., *Rickettsia*, *Chlamydia*, etc.), or highly dangerous (e.g., *B. anthracis* (agent of anthrax). The present method depends on the isolation of nucleic acid and avoids these problems since it avoids conventional bacterial isolation and characterization.

The method is expected to characterize microorganisms that have not yet been conventionally described. In addition, the present method allows distinguishing different strains of species, and this can be useful, for example, for epidemiological typing in bacteriology. The method can be used by forensic laboratories to correctly and unambiguously identify plant or animal tissues in criminal investigations. It can also be used by entomologists to quickly identify insect species, when ascertaining the nature of crop infestations.

#### Organisms Within A Genetically Related Population

The present method is useful for the determination of genetic subtypes within a specific genetically related population. That population may be represented by either prokaryotes or eukaryotes where the organisms are classified as a single genus, species, or subspecies. When identifying a prokaryotic organism, the DNA to be endonuclease-digested is that present in the cells or in the non-compartmentalized chromosomes. When identifying a eukaryotic organism nuclear DNA will be used. Preferred eukaryotic organisms that are amenable to analysis by the present method are microbial, and although may exist in an organized form such as are found in various fungal species, are generally unicellular.

Although any eukaryotic or prokaryotic organism is suitable for analysis by the instant method, the method is particularly suitable when used to analyze genera commonly associated with pathogenicity in human, animals and plants. Those genera include but are not limited to *Escherichia*, *Salmonella*, *Shigella*, *Vibrio*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Klebsiella*, *Neisseria*, *Pseudomonas* and *Campylobacter*.

#### Isolation of DNA

The present method begins with the extraction and isolation of DNA from the organism to be analyzed. Although methods of DNA extraction from both prokaryotic and eukaryotic cells is well known in the art (see for example Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)), the methodology for each is slightly different.

For prokaryotic cells, an unknown bacterium present in any medium, such as an industrial fermentation suspension, agar medium, plant or animal tissue or sample or the like, is treated under well known conditions designed to extract high molecular weight DNA. For example, cells of the unknown organism can be suspended in extraction buffer,

lysozyme added, and the suspension incubated. Cell disruption can be accelerated by addition of detergents, and/or by increase in temperature. Protease digestion followed by phenol/chloroform extraction and ethanol precipitation can be used to finalize the extraction of DNA. An alternative method of extraction, which is much faster than phenol/chloroform extraction, is rapid isolation of DNA using ethanol precipitation. This method is preferably used to isolate DNA directly from colonies or small, liquid cultures. The method is described in Davis, R. W. et al: "A Manual for Genetic Engineering, Advanced Bacterial Genetics", (hereinafter "Davis") Cold Spring Harbor Laboratory, Cold Spring, Harbor, NY, 1980, pp. 120-121, herein incorporated by reference.

Where the unknown organism is eukaryotic, an alternate methodology is used (see for example Drohan, W. et al, *Biochem. Biophys. Acta* 521 (1978) 1-15, herein incorporated by reference). Because organelle DNA is small and circular, spooling techniques serve to separate the non-circular nuclear DNA from the circular, organelle-derived DNA. As a corollary, the non-spoiled material contains the organelle-derived DNA which can separately be isolated by density gradient centrifugation. Alternatively mitochondria (or chloroplasts) are separated from a mixture of disrupted cells; the purified mitochondrial (or chloroplast) fraction is used for the preparation of organelle-derived DNA while the purified nuclear fraction is used to prepare nuclear DNA. (See for example Bonn L. and Gray, M. W., *Nucleic Acids Research* 8:319-335 (1980)).

In addition to the general method cited above DNA may be isolated from the organism to be analyzed using a variety commercial kits available from industrial sources such as Stratagene (La Jolla, CA) and Promega Corp. (Madison, WI).

#### Restriction Enzymes

The choice of restriction enzymes in the present invention is essential to the operability of the invention. Most suitable are restriction enzymes that are either 4, 5 or 6 base cutters wherein 4 base cutters are preferred. Four base cutters should have a recognition site, on average, every 256 bases along the DNA strand. Cuts at this frequency generate the large number of fragments needed for the analysis. Combinations of enzymes may also be used in a single digest where the digestion will result in the generation of nucleic acid fragments in the range of 0.1 to 18 kb. Single enzymes or combinations of enzymes that generate fragments outside of this range are not useful.

Suitable restriction enzymes useful in the present invention include but are not limited to *AccI*, *AluI*, *AvaI*, *AvaII*, *BanII*, *BfaI*, *CfoI*, *DdeI*, *DpnI*, *HaeII*, *HaeIII*, *HhaI*, *HincII*, *HinfI*, *HpaII*, *MboI*, *MnII*, *MseI*, *NciI*, *NlaIV*, *RsaI*, *Sau3AI*, *ScrFI*, *TaqI*, and *ThaI*. Methods for performing restriction digests are common and well known in the art (see for example Sambrook, J. et al., *supra*).

Selection of the appropriate enzymes for any given organism is accomplished by a process of comparing the digestions of a battery of enzymes against panel of organisms of the same taxonomic classification, epidemiologic history, or biochemical and morphologic characterization. For example, to differentiate between different strains or subtypes of a particular organism, genomic DNA is isolated from a panel of epidemiologically linked isolates as well as from a panel of epidemiologically un-linked isolates of the same genus. The isolated DNA is restricted using a large number of restriction endonucleases comprised of 4, 5 and 6 base cutters. Each enzyme employed is used in a single digestion reaction. The number of enzymes tested may vary where a panel of about 20 different enzymes (primarily 4 base cutters with some 5 and 6 base-specific cutters) is recommended. The restriction patterns or banding patterns are analyzed to identify the enzymes that will give the lowest index of diversity from the linked panel of organisms, and the highest index of diversity from the unlinked panel. The index of diversity is determined by dividing the number of banding patterns over the number of organisms tested. A pattern is considered significant if all the bands generated are less than 18 kb and discrete bands can be identified. When comparing banding patterns, the presence of only one additional band is considered significant and will represent a distinct subtype. Similarly, a difference in band intensity may define a significant variation. For example, where the banding patterns are identical with respect to the number and size of the DNA fragments, but where one or more bands differ in intensity, the difference in band intensity will be considered evidence of a distinct subtype.

Enzymes chosen on the basis of these distinctions will then be used to subtype all organisms falling into a particular species or genus.

Although frequent cutting endonucleases are effective at generating significant banding patterns, it is also possible to use more than one infrequent cutting enzyme to achieve the same effect. For example, several 6 base cutters may be used in a double, or triple digest. Depending on the organism chosen the multiple digest will generate an banding pattern of equal efficacy to that generated by a frequent base cutter. When more than one enzyme is used in inter- or intrastrain differentiation studies, their results are combined; genetic identity or clonality may then be defined as groups of independent isolates which have identical banding patterns with all the enzymes used in the study.

#### Chromatographic and Electrophoretic Methods of DNA separation

Digested DNA must be separated into a discrete banding pattern or fingerprint for subtyping to be effective. Comparison of the distinct genetic fingerprint of the organism under study with the genetic fingerprint of a specific genetic sub-type will then afford differentiation of genetic sub-types within a population of genetically related organisms.

Any method of separating DNA fragments is suitable in the present invention including HPLC, flow cytometry, membrane chromatography and gel electrophoresis. Methods of DNA separation by HPLC are common and well known in the art (see for example Wages et al., High Performance Liquid Chromatography: Principal Methods 5 Biotechnology (1996), 351-379. Editor(s): Katz, Elena D. Publisher: Wiley, Chichester, UK.; Takenaka et al., Curr. Innovations Mol. Biol. (1995), Volume Date 1995, 2, 137-54). Similarly methods of DNA separation using membrane chromatography are possible, where the DNA fragments are labeled with suitable reporters (see for example, U.S. 5,310,650 and WO 9527081). Additionally, flow cytometry has been used for separation of fluorescently 10 labeled restriction fragments (See for example Huang, et al., Nuc. Acids. Res., 24, 4202, (1996)).

The separation method of choice in the present invention is gel electrophoresis. Isolated and restricted DNA is subjected to electrophoretic separation to develop a specific and unique banding pattern. Any method of electrophoresis that is amenable to the 15 separation of nucleic acid fragments is suitable. For example, DNA fragments may be separated using gels comprised of agarose or polyacrylamide and may be non-denaturing or denaturing. The gel system may be as capillary gel electrophoresis, pulsed field gel electrophoresis, field inversion electrophoresis or 2 dimensional gel electrophoresis.

Methods for the electrophoretic separation of nucleic acids are well known in the art, 20 see for example Suvorov et al., Adv. Exp. Med. Biol. (1997), 418 (Streptococci and the Host), 979-981; Dovichi N., Autom. Technol. Genome Charact. (1997), 145-165. Editor(s): Beugelsdijk, Tony J. Publisher: Wiley, New York, NY; Caetano-Anolles, G., Fingerprinting Methods Based Arbitrarily Primed PCR (1997), 119-134. Editor(s): Micheli, Maria Rita; Bova, Rodolfo. Publisher: Springer, Berlin, Germany.

25 Preferred in the present method are agarose gels comprised of about 0.6% to about 2% agarose, where a concentration of about 1% agarose is most preferred. Gels are typically run under non-denaturing conditions and over a time period from about 4 hours to about 20 hours where a time of about 17 hours is preferred. Voltage potential across the gel may be varied to enhance fragment separation. Lower voltages of about 20v give less distinct 30 patterns as oppose to voltages in the 70v to 100v ranges. Where standard horizontal electrophoretic methods are employed it is useful to resolve the banding pattern within specific area of the gel. Generally the best results are obtained when the distance between fragments with the greatest and lowest molecular weight within the data interpretation zone (starting at the fragment with the largest molecular weight and going down to 1-2 kb 35 fragments) is in the 4 cm to 12 cm range. In a preferred embodiment it is useful to run fragments less than 2 kb off the gel to improve banding pattern resolution.

DNA Binding Agents

The present method employs a DNA binding agent for the identification of DNA bands after gel electrophoresis. Particularly suited are DNA intercalating agents capable of binding to DNA and emitting a fluorescent signal. A variety of intercalating agents are known in the art such as propidium iodide (PI) and ethidium bromide (EB) [Sailer et al., *Cytometry* (1996), 25(2), 164-172] and the cyanine dyes such as Oxazole Yellow [EP 714986], TOTO (1,1'-(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3-dihydro-(benzo-1,3-thiazole)-2-methylidene]-quinolinium tetraiodide), a homodimer of thiazole orange [Axton et al., *Mol. Cell. Probes* (1994), 8(3), 245-50] oxazole orange (YOYO)[ Srinivasan et al., *Appl. Theor. Electrophor.* (1993), 3(5), 235-9] SybrGreen™, available from Molecular Probes, Inc. (Eugene, OR, USA), and others included in U.S. Patent No. 5,563,037, hereby incorporated by reference. Preferred in the present method are ethidium bromide and propidium iodide, readily available from Sigma Chemical Company (St. Louis, MO).

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLESGENERAL METHODS

Procedures for DNA restriction analysis and other protocols common in the art of molecular biology used in the following examples may be found in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and by T. J. Silhavy, M. L. Bannan, and L. W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, pub. by Greene Publishing Assoc. and Wiley-Interscience (1987).

Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994) or in the work of Thomas D. Brock (in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA). All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals



(Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

Restriction enzymes used in the following examples were obtained from American Allied Biochemicals (Aurora, CO), New England Biolabs (Beverly, MA) and Boehringer-Mannheim (Indianapolis, IN).

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "uL" means microliters, "mL" means milliliters, and "L" means liters.

#### Bacterial strains

10 *Escherichia coli* O157:H7 strains were obtained from Washington State Health Department, King County laboratories, and Center for Disease Control and Prevention and grown on tryptic soy agar (Difco Laboratories).

#### Growth Conditions

15 All strains were grown overnight on tryptic soy agar. Confluent bacterial growth on agar plates was scraped with several sweeps of a sterile flat-headed toothpick and suspended in 0.8 mL of 50 mM Tris (pH 8.0) containing 50 mM EDTA. Forty-five uL of 20% sodium dodecyl sulfate (SDS) and 10 uL of Proteinase K (20 mg/mL; Boehringer-Mannheim, Indianapolis, IN) was added to all suspensions. The suspensions were incubated at 50°C for 1 h.

#### 20 Isolation of DNA

##### DNA Isolation from Gram Negative Bacteria:

Confluent bacterial growth on agar plates was scraped with several sweeps of a sterile flat-headed toothpick and suspended in 0.8 mL of 50 mM Tris, 50 mM EDTA (pH 8.0). Forty-five uL of 20% sodium dodecyl sulfate (SDS) and 10 uL of proteinase K (20 mg/mL ; Pharmacia, Piscataway, N.J.) was added to all suspensions. The suspensions were incubated at 50°C for 1 h. Phenol-chloroform and chloroform extractions are performed on the DNA suspensions. The DNA is precipitated with the addition of 2.5 times the volume of absolute ethanol, spooled with a glass capillary pipette and suspended in 100-200 uL sterile dH<sub>2</sub>O.

##### 30 DNA Isolation from Gram Positive Bacteria:

Confluent bacterial growth on agar plates was scraped with several sweeps of a sterile flat-headed toothpick and suspended in 0.8 mL of 50 mM Tris, 50 mM EDTA (pH 8.0), plus 10 mg/mL lysozyme and incubated for 1 h, at 37°C. Fifty uL of 5 mg/mL solution of Lysostaphin (Sigma, St. Louis, MO) was added to each tube and the mixture was incubated for 1 h, at 37°C. Forty-five uL of 20% sodium dodecyl sulfate (SDS) and 10 uL of proteinase K (20 mg/mL; Pharmacia, Piscataway, NJ) was added to all suspensions. The suspensions were incubated at 50°C for 1 h. Phenol-chloroform and chloroform extractions

are performed on the DNA suspensions. The DNA is then precipitated with the addition of 2.5 times the volume of absolute ethanol, spooled with a glass capillary pipette and suspended in 100-200  $\mu$ L sterile dH<sub>2</sub>O.

### Restriction Digests:

5 Ten to twenty microliters from each DNA preparations (1 ug/uL) was digested with 10-20 units of appropriate restriction enzymes in a 30 uL reaction volume according to the manufacturer's instructions. All DNA preparations were digested for a minimum of 2 h at 37°C (unless other temperatures were indicated by the manufacturer).

## Electrophoresis

10      Following digestion, DNA fragments were electrophoretically separated on 15x20 cm agarose gels in 1X Tris-borate EDTA. Unless otherwise specified the gels were 1% agarose. Unless otherwise specified the gels were run for 17 h at 70 v. Lambda-Hind III and 1KB ladder were used as the molecular size markers. Gels were stained with ethidium bromide for 20 min, destained with deionized water for 1 h, and visualized.

15 EXAMPLE 1  
SUBTYPING OF *E. COLI* 0157

Example 1 demonstrates the process for the selection of appropriate restriction enzymes for the subtyping of a particular organism and the subsequent determination of a genetic subtype.

Two panels of *E. coli* O157 isolates with known epidemiological history were assembled. In order to identify appropriate restriction enzymes, groups of *E. coli* O157:H7 strains were analyzed with various enzymes. The results are shown in Figures 1a-m.

Genomic DNA was isolated and extracted from the members of each panel according to the protocol described in the GENERAL METHODS. Extracted DNA was then restricted with a variety of enzymes listed below:

*AluI, AscI, Aval, Avall, BamHI, BfaI, Bstul, BspI, DdeI, DpnI, EcoRI, HincII, HindIII, HaeIII, HinFI, HhaI, MnlI, MspI, NciI, nlaIII, NlaIV, PvuII, RsaI, Sau3AI, Scrfl, TaqI.*

Restriction digested proceeded according to the method described in the GENERAL METHODS, and the restriction fragments were resolved by gel electrophoresis, with the exception that the voltage was varied between 22-34 volts (Figures 1a-m). The resulting banding patterns were analyzed to identify the restriction enzymes which could potentially generate epidemiologically relevant data. Enzymes generating DNA fragments no larger than 18 kb, where the majority of bands are less than 12 kb are selected for further use. As can be seen in Figures 1a-l, 15 of the 26 enzymes have banding patterns which start under 18 kb.

Upon further examination, of the 14 enzymes, several were excluded and due to lack of sensitivity in inter-strain differentiation of *E. coli* O157:H7, or the nature of their banding

patterns. For example *AvaII* was excluded because it generated a very compressed banding pattern in the 18 kb size range. *HinfI* and *NlaIII* were excluded because almost all the bands were below 4 kb which resulted in a very compressed and indecipherable banding pattern.

5 Several of the enzymes were selected for further testing results are shown in Figures 2a-i. From this smaller set of enzymes, *HaeIII* and *Sau3AI* were selected as the primary and secondary enzymes to be used for *E. coli* O157:H7 subtyping.

DNA from a panel of unlinked isolates were restricted with *HaeIII*, *Sau3AI*, *NciI* and *AluI* and the fragments resolved by gel electrophoresis (Figure 2i). As can be seen by the data, *Sau3AI* and *HaeIII* were clearly superior to *NciI* and *AluI* in inter-strain differentiation of *E. coli* O157:H7. It is also clear that *NciI* and *AluI* may be used in situations where more genomic characterization is needed. Figures 3a-b demonstrate the use of *Sau3AI* and *Hae3A* on a panel of epidemiologically linked and unlinked isolates. Lanes 1, 3, and 4 in each figure represent epidemiologically unlinked isolates, and lanes 2, 5, 6, 7, and 8 represent epidemiologically linked strains. Each enzyme separates the unlinked isolates from each other and from the linked ones. Each of the two enzymes gave identical banding patterns for the members of the linked panel.

## EXAMPLE 2

### EFFECT OF RESTRICTION FRAGMENT SIZE ON PATTERN SPECIFICITY

20 Example 2 compares use of restriction enzymes which produce banding patterns below 18 kb (frequent cutters) over enzymes with less frequent recognition sites (generally 6 base cutters) in the present method.

*E. coli* O157 cells from three different strains were prepared as described in Example 1. Genomic DNA was extracted and separated into four pools. One pool was restricted with the 4 base cutter *Sau3AI* and the other pools with the 6 base cutters *EcoRI*, *HindIII* and *PvuII*. The resulting fragment separation is illustrated in Figure 4.

As can be seen by the data, the banding patterns generated by the 6 base cutters are unable to generate a significant banding fingerprint. In contrast, the banding pattern generated by *Sau3AI* is very informative and easy to interpret. The three strain which had no epidemiological connection show banding patterns which are different from each other.

## EXAMPLE 3

### BACTERIAL SUBTYPING BY DOUBLE DIGEST WITH RARE CUTTING ENDONUCLEASES

35 Example 3 illustrates that rare cutting restriction endonucleases, when used in combination, have the ability to produce a significant banding fingerprint, similar to that achieved by using frequent cutting enzymes.

*E. coli* 0157 cells were prepared as described in Example 1. Genomic DNA was extracted and separated into 3 pools. One pool was restricted with *SmaI* and *HincII*, the second pool was cut with *PstI* and *HindIII*, and the third was restricted using *HindIII* and *EcoRV*. As it is evident from Figure 5 combination of *HincII* and *SmaI* resulted in generating fragments less than 18 kb in a significant banding fingerprint.

#### EXAMPLE 4

##### EFFECT OF DNA CONCENTRATION ON RESTRICTION PATTERN

Example 4 demonstrates the effect of starting concentration of DNA on the resolution of restricted fragments by gel electrophoresis.

*E. coli* 0157 cells were prepared as described in Example 1. Genomic DNA was extracted and aliquoted for restriction digest. 1 ug, 2 ug, 4 ug, 8 ug, 16 ug, and 20 ug of DNA were restricted in separate reactions using *HaeIII* and *Sau3AI*. The resulting banding patterns were resolved by gel electrophoresis according to the protocol in the GENERAL METHODS. The results are illustrated in figure 6: Lane 1, 1' kb ladder; lane 2, *Lambda-HindIII*, Lanes 3-8, 1 ug, 2 ug, 4 ug, 8 ug, 16 ug, and 20 ug of *E. coli* 112 cut with *HaeIII*, lanes 9-14 same quantities of DNA cut with *Sau3AI*.

As can be seen by the data in Figure 6, banding patterns are seen clearly at 16-20 ug range. In contrast 1-2 ug of DNA is sufficient to obtain a banding pattern with 6 base cutters; however, as has been demonstrated, such cutters used alone are not effective in the present method.

#### EXAMPLE 5

##### EFFECT OF AGAROSE VARIATION ON THE RESOLUTION OF BANDING PATTERNS

DNA was prepared as described above from 3 different panels of *E. coli* O157:H strains. DNA from two of the panels were restricted with *NciI* and *Sau3AI* and applied to gels with varying concentrations of agarose. All gels were run for 17 hr and at 45 volts. Figures 7a and b illustrate the difference in separation when the agarose concentration is varied between 1% and 2%. As can be seen by the data, more concentrated agarose produces more compressed banding patterns which will limit the utility of the method.

DNA from the third panel was separated into 3 pools; each pool was digested using *Sau3AI*. The three pools were applied to three different agarose gels with agarose concentrations of 0.7%, 0.8% and 1% (Figures 8a, 8b, and 8c, respectively). Electrophoresis was conducted for 17 h, at 45 volts. As is evident from the data, the resolution improves with lower concentrations of agarose. However, the smaller bands (around 3-4 kb and lower) become noticeably diffused.

## EXAMPLE 6

### DEMONSTRATION OF METHOD ON VARIETY OF BACTERIA

Example 6 demonstrates that the present method of bacterial subtyping is applicable to a diverse number of gram positive and gram negative bacterial genera.

5 All strains were grown according to the protocols in the GENERAL METHODS and DNA was extracted and prepared for restriction analysis. Genomic DNA was extracted from *Listeria*, *Salmonella*, *Shigella*, *E. coli*, *Klebsiella*, *Pseudomonas*, *Vibrio*, *Staphylococcus*, and *Neisseria*.

#### Salmonella and Shigella

10 To identify the enzymes that produce banding patterns in the desired size range (less than 18 kb), a panel of three strains was assembled, consisting of one *E. coli* 0157:H (#1706), one *Shigella sonnei* (#14), and one *Salmonella bovis* (#66). Isolates were grown, and DNA was isolated as described in the general methods section. DNA was restricted with *AviI*, *Bst*II, *DpnI*, *HhaI*, *MspI*, *Nla*III, *Nla*IV, *RsaI*, and *SacI*. The results are  
15 shown in Figures 9a and 9b. A separate panel of *S. sonnei* isolates were tested by *Hae*III, *Nci*I and *Sau*3AI. *S. sonnei* isolates are shown in Figure 9c, and *Salmonella* isolates are in Figure 9d.

Figures 10a and 10b show the use of *Sau*3AI and *Hae*III on a panel of *S. sonnei* isolates of known epidemiological history (containing linked and unlinked cases). *Sau*3AI  
20 alone divided the isolates into appropriate epidemiological groups (Figure 10b). Although *Hae*III showed less sensitivity, it demonstrated good differentiative power, qualifying it as one of the secondary enzymes for *Shigella*. Several of the other enzymes including *Nci*I were also deemed to be useful in the study of *Shigella* spp.

A panel of 11 *Salmonella bovis* isolates of known epidemiological  
25 history were assembled. The isolates were grown, and DNA was purified as described above. The DNA preps were restricted with *Hae*III (Figure 11a), *Alu*I (Figure 11b), *Nci*I (Figure 11c), and *Sau*3AI (Figure 11d). Both *Sau*3AI (Figure 11d) and *Nci*I (Figure 11c) showed that lanes 5-13 had identical banding patterns, and lanes 3 and 4 each had unique patterns. The data confirmed the epidemiological data which showed that strains 5-13 were  
30 from a single cluster and strains 3 and 4 were unlinked isolates. *Alu*I could not separate the unlinked isolates from each other and from the linked isolates. *Hae*III separated the unlinked from the linked isolates but it could not separate them from each other.

#### Neisseria

A panel of *Neisseria meningitidis* isolates was grown, DNA was isolated (as  
35 described in the GENERAL METHODS), and tested with *Asc*I, *Bfa*I, *Dpn*II, *Hae*III, *Mn*II, and *Sau*3AI (*Dpn*II and *Sau*3AI have identical recognition sequences). The results indicate that *Hae*III, and *Sau*3AI/*Dpn*II were equally useful for *Neisseria* species (Figure 12a).

Figure 12b shows the use of *Sau3AI*, *HaeIII* and *MnlI* on two independent isolates of *Neisseria meningitidis*.

*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Vibrio parahaemolyticus*

On the basis of the previous results for other gram negatives (*E. coli*, *Salmonella*  
5 *bovismorbificans* and *Shigella sonnei*), *Sau3AI*, *HaeIII*, and *NciI* were chosen to be tested on *Pseudomonas aeruginosa*. The data is shown in Figure 12c. *NciI* was selected as one of the primary enzymes for *Pseudomonas aeruginosa*. Figure 12d shows the use of *NciI* on a panel of *P. aeruginosa* isolates of known epidemiological history. The enzyme separated the linked and the unlinked isolates from each other and the subtyping data 100% matched the  
10 epidemiological data.

Figure 13a shows a panel of *Klebsiella pneumoniae* isolates with known epidemiological history tested with *HaeIII*, and *Sau3AI*. As can be seen by the data, both enzymes divided the group according to the epidemiological linkages.

Figures 13b and 13c show the utility of the method in interstrain differentiation of  
15 *Vibrio parahaemolyticus*. A panel of *V. parahaemolyticus* isolates with known epidemiological history was analyzed with *Sau3AI* and *HaeIII*. Both enzymes proved to be very powerful in differentiation of the *V. parahaemolyticus* isolates. Combination of the results of the two enzymes showed the true linkages of the isolates.

*Enterococci spp.*

On the basis of the results obtained with other gram positives (*Staphylococcus aureus*  
20 and *Listeria monocytogenes* - see below), a panel of *Enterococci* isolates with known epidemiological history was obtained and tested with *HaeIII*, and *HhaI*. *HaeIII* and *HhaI* resulted in epidemiologically relevant data (Figures 14a and 14b). The electrophoresis conditions were revised to employ a 6% agarose gel for analysis of *Enterococci* with *HaeIII* and *HhaI* (Figure 14c).  
25

*Staphylococcus aureus*

Two panels of *Staphylococcus aureus* strains of known epidemiological history were assembled. Cells were grown and DNA was isolated as described above. One member of the panel was tested with a panel of restriction enzymes. The data is shown in Figure 15a where  
30 Lane 1, is 1 kb ladder, lanes 2-14 are *Staphylococcus aureus* DNA cut with *AluI*, *AscI*, *AvaI*, *AvaII*, *BfaI*, *Bstul*, *DdeI*, *DpnII*, *MnlI*, *MseI*, *MspI*, *RsaI*, and *ScrFI*, respectively. *Sau3AI*, *HaeIII*, *HhaI*, *NciI*, and *NlaIV* were also tested with a *Staphylococcus aureus* isolate (data not shown).

Several of the enzymes used above generated the banding of appropriate size range  
35 (starting at or below 18 kb). Figures 15b-f show the use of *HaeIII*, *NlaIV*, *HhaI*, *AluI*, and *DdeI* respectively. The results obtained from each one of the enzymes validated the epidemiological linkages which were available for the panel. *DdeI*, *MnlI*, and *HhaI* were

used on the second panel of *Staphylococcus* isolates. The results are shown in Figures 15g and 15h, again the epidemiological information matched the typing data generated by the enzymes.

#### *Listeria monocytogenes*

5 A panel of *Listeria* strains of known epidemiological history were assembled, cells were grown, and DNA was extracted as described above. To determine the appropriate enzymes, *Listeria monocytogenes* DNA was restricted with a panel of enzymes. Figure 16a shows the results. Lane 1, 1KB ladder, lanes 2-14 are *Listeria monocytogenes* DNA restricted with *AscI*, *AvaI*, *AvaII*, *BfaI*, *BstI*, *DdeI*, *DpnI*, *MnlI*, *MseI*, *MspI*, *NlaIV*, *RsaI*,  
10 and *ScrFI*, respectively.

*Listeria monocytogenes* DNA was separately tested with *HaeIII*, *AluI*, *Sau3AI*, *HhaI*, *DpnII*, and *NciI* (data not shown). Several of the enzymes generated the desired banding size range (starting at or below 18KB). Based on these tests *DpnII* was selected as an appropriate enzyme for subtyping. Figure 16b shows 12 *Listeria monocytogenes* isolates of known  
15 epidemiologic history restricted with *DpnII* showing a distinguishable fingerprint.

## WHAT IS CLAIMED:

1. A method for the differentiation of genetic sub-types within a population of genetically related organisms comprising the steps of:
  - (i) isolating genomic DNA from an organism to be analyzed;
  - 5 (ii) restricting genomic DNA from said organism with at least one restriction endonuclease to yield restricted DNA wherein all fragments are less than 18kb;
  - (iii) resolving said restricted DNA by chromatographic means capable of separating fragments in the 0.1 to 18 kb range to generate a distinct genetic  
10 fingerprint;
  - (iv) comparing said distinct genetic fingerprint of step (iii) with the genetic fingerprint of a specific genetic sub-type wherein differences in said genetic fingerprints afford differentiation of genetic sub-types within a population of genetically related organisms.
- 15 2. A method according to Claim 1 wherein said organism is selected from the group consisting of prokaryotes and microbial eukaryotes.
3. A method according to Claim 2 wherein said organism is selected from the group consisting of gram positive bacteria and gram negative bacteria.
4. A method according to Claim 3 wherein said organism is selected from  
20 the group consisting of *Escherichia*, *Salmonella*, *Shigella*, *Vibrio*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococci*, *Klebsiela*, *Nicericia* *Pseudomonas* and *Campylobacter*.
5. A method according to Claim 4 wherein said organism is selected from the group consisting of *E. coli* 0157 and *Salmonella*.
- 25 6. A method according to Claim 1 wherein said chromatographic means of step (iii) is selected from the group consisting of gel electrophoresis, HPLC, flow cytometry, and membrane chromatography.
7. A method according to Claim 6 wherein said method of gel electrophoresis is selected from the group consisting of capillary gel electrophoresis,  
30 pulsed field gel electrophoresis, 2 dimensional gel electrophoresis, and field inversion electrophoresis.
8. A method according to Claim 7 wherein said gel is an agarose gel having a concentration of from about 0.6% to about 2% agarose.
9. A method according to Claim 7 wherein said gel is polyacrylamide.
- 35 10. A method according to Claim 7 wherein said restricted DNA is stained with a DNA binding dye selected from the group consisting of ethidium bromide, propidium iodide, and fluorescent cyanine dyes.



11. A method according to Claim 1 wherein said at least one restriction enzyme is selected from the group consisting of *AccI*, *AluI*, *AvaI*, *AvaII*, *BanII*, *BfaI*, *CfoI*, *DdeI*, *DpnI*, *HaeII*, *HaeIII*, *HhaI*, *HincII*, *HinfI*, *HpaII*, *MboI*, *MnII*, *MseI*, *NciI*, *NlaIV*, *RsaI*, *Sau3AI*, *ScrfI*, *TaqI*, and *ThaI*.
- 5 12. A method according to Claim 1 wherein said at least one restriction enzyme is a 4 base cutter.
13. A method according to Claim 1 wherein said at least one restriction enzyme is a combination of at least two 6 base cutters.
14. A method according to Claim 1 wherein said genomic DNA is from  
10 about 5 ug to about 30 ug.
15. A method according to Claim 1 wherein said resolving of restricted DNA at step (iii) results in the elimination of all DNA fragments less than 2 kb.
16. A method for the interstrain differentiation of bacterial isolates comprising the steps of:
- 15 (i) isolating genomic DNA from a) a panel of epidemiologically linked bacterial isolates and b) a panel of epidemiologically un-linked bacterial isolates wherein said linked and un-linked isolates are members of the same genus;
- (ii) restricting genomic DNA from the linked and un-linked panel of isolates with a panel of restriction enzymes selected from the group consisting of  
20 *AccI*, *AluI*, *AvaI*, *AvaII*, *BanII*, *BfaI*, *CfoI*, *DdeI*, *DpnI*, *HaeII*, *HaeIII*, *HhaI*, *HincII*, *HinfI*, *HpaII*, *MboI*, *MnII*, *MseI*, *NciI*, *NlaIV*, *RsaI*, *Sau3AI*, *ScrfI*, *TaqI*, and *ThaI*;
- (iii) resolving the restricted DNA of step (ii) by gel electrophoresis to generate a banding pattern for each enzyme;
- (iv) selecting at least one enzyme from the panel of enzymes of step (ii)  
25 that a) restricts all of the genomic DNA to fragments of less than 18 kb, b) generates the lowest index of diversity from the linked panel and c) generates the highest index of diversity from the unlinked panel;
- (v) restricting the isolated DNA from the linked and un-linked panels of bacterial isolates of step (i) with the at least one enzyme of step (iv);
- 30 (vi) resolving said restricted DNA of step (v) by gel electrophoretic means capable of separating fragments in the 0.1 to 18 kb range to generate a distinct genetic fingerprint for said linked and unlinked isolates; and
- (vii) comparing said distinct genetic fingerprints of step (vi) wherein differences in said genetic fingerprints affords interstrain differentiation of said  
35 bacterial isolates.
17. A method according to Claim 16 wherein said method of gel electrophoresis is selected from the group consisting of, capillary gel electrophoresis,

pulsed field gel electrophoresis, 2 dimensional gel electrophoresis, inversional electrophoresis and vertical electrophoresis.

18. A method according to Claim 16 wherein said gel is an agarose having a concentration of from about 0.6% to about 2% agarose.

5 19. A method according to Claim 16 wherein said gel is polyacrylamide.

20. A method according to Claim 16 wherein said restricted DNA is stained with a DNA binding dye selected from the group consisting of ethidium bromide, propidium iodide, and fluorescent cyanine dyes.

10 21. A method according to Claim 16 wherein said at least one restriction enzyme is a 4 base cutter.

22. A method according to Claim 16 wherein said at least one restriction enzyme is a combination of at least one 4 base cutter and at least one 6 base cutter.

23. A method according to Claim 16 wherein said genomic DNA is from about 5 ug to about 30 ug.

15 24. A method according to Claim 16 wherein said resolving of restricted DNA at step (iii) results in the elimination of all DNA fragments less than 2 kb.

25. A method according to Claim 16 wherein said resolving of restricted DNA at step (iii) results in separation of DNA fragments over an distance of about 4 cm to about 12 cm of gel.

20 26. A method according to Claim 16 wherein said bacterial isolate is selected from the group consisting of *Escherichia*, *Salmonella*, *Shigella*, *Vibrio*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococci*, *Klebsiela*, *Nicericia* *Pseudomonas* and *Campylobacter*.

25 27. A method according to Claim 16 wherein said bacterial isolate is selected from the group consisting of *E. coli* 0157 and *Salmonella*.

## DECLARATION and POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**METHOD FOR THE IDENTIFICATION OF GENETIC SUBTYPES**

the specification of which is attached hereto unless the following box is checked:

☒ was filed on 9 March 1998 as U.S. Application No. \_\_\_\_\_ or PCT International Application No. PCT/US98/04599 and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known to me to be material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Application No.	Country	Filing Date	Priority Claimed (Yes/No)
I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States Provisional Application(s) listed below.			
U.S. Provisional Application No.		U.S. Filing Date	
60/039,056		MARCH 10, 1997	

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International Application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT International Application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is known to me to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application No.	Filing Date	Status (patented, pending or abandoned)
PCT/US98/04599	MARCH 9, 1998	Pending

**POWER OF ATTORNEY:** I hereby appoint the following attorney(s) and/or agent(s) the power to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:Name: WILLIAM R. MAJARIANRegistration No.: 41,17355

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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☐ Additional Inventors are being named on separately numbered sheets attached hereto.

**GENERAL POWER OF ATTORNEY**  
(Concerning Several International Patent Applications)

The undersigned, Howard J. Rudge, Senior Vice President and General Counsel of E. I. DU PONT DE NEMOURS AND COMPANY, 1007 Market Street, Wilmington, Delaware 19898 USA ("DuPont"), hereby confirms that the power to sign for DuPont has been granted to various individuals (as set forth in the attached excerpt from DuPont's Patent Board Rules of Procedure (January 1988), Appendix Section III.A.4), including the Chairman, Vice-Chairman, and those individuals who are Assistant Secretaries of the Patent Board. Currently these Assistant Secretaries are:

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In addition, the authority to act on behalf of DuPont before the competent International Authorities in connection with any and all international patent applications filed by it with the United States as Receiving Office and to make or receive payments on its behalf is hereby granted to:

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The undersigned ratifies fully all actions already taken by the above-named individuals in accordance with the authority granted hereby.

E. I. DU PONT DE NEMOURS AND COMPANY

By: Howard J. Rudge  
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Senior Vice President and General Counsel

Date: July 15, 1999